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(54) DETECTION OF ENTEROVIRUS AND DISCRIMINATION OF THE SAME

(57)Abstract:

PURPOSE: To detect Picornaviridae such as Enterovirus, etc., by amplifying a Specific region of Enterovirus and detecting amplified gene DNA.

CONSTITUTION: An oligonucleotide (e.g. CTACTTGGGTGTCCGTGTT) having complementarity to a common type part in the upstream of a gene region coding a part of 5'-non-translated region of Enterovirus, a part of VP4 and VP2 proteins, and an oligonucleotide (e.g.

TGGTGGTGGAAAGTTGCCTGA) having complementarity to a common type part in the downstream are subjected to be primers of the PCR method. The amplified gene DNA is detected by polyacrylamide gel electrophoresis, etc.

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CLAIMS

[Claim(s)]

[Claim 1] (i) The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of enterovirus, and some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. The method of detecting the enterovirus characterized by amplifying the gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in a part of 5'-untranslation region of enterovirus, and the human serum protein type of enterovirus, and detecting the (ii) this magnification gene DNA.

[Claim 2] (i) A part of 5'-untranslation region of an enterovirus separation stock with a strange human serum protein type The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. The gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a strange enterovirus separation stock and the human serum protein type of enterovirus is amplified. This magnification gene DNA is solid-phase-ized to a microplate. A part of 5'-untranslation region of the epidemic enterovirus separation stock of known [human serum protein type / (ii)] The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. And carry out an indicator and it considers as the DNA probe for human serum protein type discernment. the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a known epidemic enterovirus separation stock, and the human serum protein type of enterovirus -- magnification -- (iii) this DNA probe -- the DNA solid phase-ized microplate of the above (i) -- in addition, the human serum protein type discernment approach of the enterovirus which is made to carry out hybridization under **** conditions, and is characterized by analyzing the class of joint probe.

[Claim 3] (i) The oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of enterovirus and some ****4 and ****2 proteins is the following array (1).

CTACTTGGGTGTCCGTGTT (1)

The oligonucleotide which comes out, has the base sequence shown and has a complementarity in a down-stream mold intersection is the following array (2).

TGGTGGTGGAAAGTTGCCTGA (2)

Detection or the discernment approach of claim 1 characterized by being the oligonucleotide which comes out and has the array shown, or the enterovirus of 2.

[Translation done.]

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DETAILED DESCRIPTION

[Detailed Description of the Invention]**[0001]**

[Industrial Application] This invention detects enterovirus to high sensitivity, and relates to the approach of identifying a human serum protein type.

[0002]

[Description of the Prior Art] It is difficult to presume the virus which the enterovirus (Enterovirus) belonging to the Picornaviridae (Picornaviridae) is classified into about 70 kinds of human serum protein types, and the rhinovirus (Rhinovirus) which similarly belongs to the Picornaviridae is classified into about 100 kinds of human serum protein types, shows a variegated infectious disease, and becomes a cause from a clinical manifestation. Therefore, separation identification of a virus is needed for deciding a pathogen. However, a current enterovirus separation method of identification separates a virus using cultivation, and the protection test is further needed for identification. And two - four weeks is required for the isolation culture of these viruses. The separation stock which the protection test which furthermore used the neutralization antiserum of a standard stock cannot human serum protein type judge appears frequently. This is considered for the gene of enterovirus to vary extremely in a nature at high speed, and production of the antiserum which always neutralizes a fresh separation stock is needed for these solutions. In chlamydia (Chlamydia), the approach of detecting for a short time, using a DNA probe as a direct detection method of the pathogen of an infectious disease is established. However, the detection sensitivity is low, and in enterovirus, in order that the amount of viruses required for the probe method may not be obtained from a patient specimen but the gene of enterovirus may vary to a high speed extremely still like previous statement, the difficulty of identification is expected with the oligo probe of a standard stock. High sensitivity and polymerase chain reaction method [Polymerase Chain Reaction which amplifies DNA specifically Law, ; which writes this as the "PCR method" below, after Saiki et al., Science, 230 volumes, p1350-1354, and 1985 reference] are developed the PCR method using a primer complementary to the base sequence of a 5'-untranslation region, and 5' -- by the PCR method using the primer which has a complementarity in the base sequence of the gene field which carries out the code of the ****4 and ****2 protein in - untranslation region [Rotbart. by which enterovirus is detected H. and S.J. -- Clinical microbiology. and 28 438-442 (1990); Olive.D., M., S.J. general Virology., 71, and 2141-2147 (1990) --]. However, these approaches cannot identify the human serum protein type of enterovirus, therefore enterovirus is detected in a higher precision and the approach of judging a human serum protein type is searched for.

[0003]

[Problem(s) to be Solved by the Invention] This invention aims at offer of the approach of judging the human serum protein type of enterovirus in a high precision while it can detect picornaviruses, such as enterovirus and rhinovirus, in a high precision.

[0004]

[Means for Solving the Problem] this invention persons have a specific base sequence in a part of 5'-untranslation region of enterovirus, and the human serum protein type of enterovirus. Detection [high

sensitivity picornaviruses /, such as enterovirus,] is possible by amplifying a field including the gene field which carries out the code of some ****4 and ****2 proteins, and detecting this magnification gene DNA, Furthermore, a human serum protein type uses a known epidemia enterovirus separation stock for this magnification gene DNA, and the same field as the above is combined under the produced DNA probe which amplified and carried out the indicator, and **** conditions. By detecting the joint indicator DNA and analyzing the class of united probe, it finds out that discernment of the highly precise human serum protein type of enterovirus is possible, and came to complete this invention.

[0005] According to this invention, in this way A part of 5'-untranslation region of 1. (i) enterovirus The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. The gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in a part of 5'-untranslation region of enterovirus and the human serum protein type of enterovirus is amplified. (ii) The method of detecting the enterovirus characterized by detecting this magnification gene DNA, 2. A part of 5'-untranslation region of an enterovirus separation stock with the strange (i) human serum protein type The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. The gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a strange enterovirus separation stock and the human serum protein type of enterovirus is amplified. This magnification gene DNA is solid-phase-ized to a microplate. A part of 5'-untranslation region of the separation stock of the epidemia enterovirus of known [human serum protein type / (ii)] The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. And carry out an indicator and it considers as the DNA probe for human serum protein type discernment. the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a known epidemia enterovirus separation stock, and the human serum protein type of enterovirus -- magnification -- A DNA probe is added to the DNA solid phase-ized microplate of the above (i). (iii) The human serum protein type discernment approach of the enterovirus which is made to carry out hybridization under **** conditions, and is characterized by analyzing the class of joint probe, 3. The oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of (i) enterovirus and some ****4 and ****2 proteins is the following array (1).

CTACTTGGGTGTCCGTGTT (1)

The oligonucleotide which comes out, has the base sequence shown and has a complementarity in a down-stream mold intersection is the following array. TGGTGGTGGAAGTTGCCTGA (2)

Detection or the discernment approach of of the above 1 or the enterovirus of 2 characterized by being the oligonucleotide which comes out and has the base sequence shown is offered.

[0006] Detection and the discernment approach of the enterovirus of this invention are further explained to a detail below. a particle symmetrical with the regular icosahedron of the ether resistance in which "picornavirus" does not have an envelope in this specification -- it is -- the diameter of 20-30nm -- it is -- a core -- single stranded RNA -- having -- the molecular weight of this RNA -- about 2.5×10^6 it is -- the virion which has infectivity and has the function of mRNA is meant. moreover, "enterovirus" -- the above-mentioned Picornaviridae -- belonging -- and pH3.0 -- stable -- the buoyant density in the inside of CsCl -- 1.32-1.35g/cm³ it is -- virion is meant and the Coxsackie A group virus, the Coxsackie B group virus, echovirus, enterovirus, a poliovirus, etc. are included by this enterovirus. further -- "rhinovirus" -- the above-mentioned Picornaviridae -- belonging -- and pH3.0 -- unstable -- the buoyant density in the inside of CsCl -- 1.38 - 1.40 g/cm² it is -- virion is meant. One description of this invention is to identify the human serum protein type of this enterovirus while detecting enterovirus by analyzing the class of probe which the human serum protein type increased a part of gene of the strange

enterovirus separation stock origin, made combine by the hybridization under the DNA probe and the **** conditions that magnification and the human serum protein type which carried out the indicator produced the same field from the gene of a known epidemia enterovirus separation stock, and combined. Such an approach enables it to identify the human serum protein type of enterovirus, while enterovirus is detectable in a high precision.

[0007] Since a close relationship [a human serum protein type / between those with about 70 sorts, and each human serum protein type], as for enterovirus, it is desirable to use the hybridization under the **** conditions which discernment of a human serum protein type is difficult, and are used by this invention on the occasion of discernment of a human serum protein type on the usual hybridization conditions. Here, the hybridization under **** conditions means the hybridization under existence of a formamide. Especially the abundance of the formamide in this hybridization condition usually has 40 - 60% of desirable within the limits 20 to 70%, and especially reaction temperature has desirable within the limits of 40-60 degrees C 40-70 degrees C. Although there is especially no limit in reaction time, within the limits of 1 - 24 hours is usually suitable. Although a standard stock and a separation stock (they are a vaccine stock and a decomposition stock in the case of a poliovirus) will be distinguished in the same human serum protein type and discernment of the human serum protein type of a separation stock is impossible in the hybridization under the above-mentioned **** conditions As a source of enterovirus gene DNA for the DNA probe creation for human serum protein type discernment The DNA probe for human serum protein type discernment by which the human serum protein type was created using the known epidemia enterovirus separation stock (namely, enterovirus stock which was in fashion and was separated within the past ten years) is used. It becomes discriminable [detection of each enterovirus, and a human serum protein type] by performing hybridization under the above-mentioned **** conditions, and analyzing a joint pattern.

[0008] Magnification of a gene field including the human serum protein type specific base sequence of enterovirus, i.e., "the gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in a part of 5'-untranslation region of enterovirus and the human serum protein type of enterovirus", can be performed as follows. First, the isolation culture stock from clinical specimens, such as cerebrospinal fluid extracted at the time of a medical examination, and a clinical specimen and the human serum protein type by which subculture is carried out extract RNA from a known enterovirus standard stock etc. with a conventional method, and produces cDNA for this extract RNA using reverse transcriptase. The die length which includes the gene field which carries out the code of the 5'-untranslation region of enterovirus, and ****4 and ****2 for the oligonucleotide which has a human serum protein type specific base sequence, i.e., "the oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of an enterovirus separation stock and some ****4 and ****2 proteins, and a down-stream mold intersection", as a primer in this cDNA amplifies the gene DNA field of about 650 bases. the PCR method for which magnification of a gene is usually used -- [-- JP,61-274697,A, JP,62-281,A, 239 Sakai sScience(s), and p487-491 reference] can perform the detail of this PCR method easily.

[0009] On the occasion of magnification of a gene field including the human serum protein type specific base sequence of enterovirus, as an oligonucleotide which can be used as a primer The oligonucleotide which has a complementarity in the mold intersection of the upstream of a gene field including a human serum protein type specific base sequence, and a down-stream mold intersection, Namely, if "the oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of enterovirus and some ****4 and ****2 proteins, and a down-stream mold intersection" is used for coincidence You may be what kind of oligonucleotide. It is appropriate to use as a primer the oligonucleotide which was specific to enterovirus, and set the high base sequence of similarity as the 5'-untranslation region (upstream mold intersection) and ****2 field (down-stream mold intersection) between seeds, and carried out chemosynthesis in them based on the base sequence based on desirable known human serum protein type specific base sequence data.

[0010] As the primer which carried out chemosynthesis, i.e., an oligonucleotide which has a complementarity in the mold intersection of the upstream of an enterovirus specific gene field, it is the following array (1).

CTACTTGGGTGTCCGTGTT (1)

The oligonucleotide which has a complementarity in a down-stream mold intersection is the following array (2).

TGGTGGTGGAAAGTTGCCTGA (2)

It is more desirable to use the primer which comes out and has the base sequence shown. The chemosynthesis of the primer mentioned above is model 381-A, the known the nucleic-acid-biosynthesis machine usually used, for example, Applied Biosystem make, in itself. It can carry out easily with the solid phase synthesis method using a DNA synthesis machine etc. Like the above, polyacrylamide gel electrophoresis, agarose gel electrophoresis, etc. which are usually used can separate, and the gene field DNA including the human serum protein type specific base sequence of the enterovirus which was carried out and was amplified by the PCR method can be detected as a band, and, thereby, can check the gene DNA of the enterovirus origin. In addition, detection of the DNA band after electrophoresis can be dyed by the ethidium bromide, and UV irradiation can perform it easily.

[0011] DNA of "the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a strange enterovirus separation stock and the human serum protein type of enterovirus" obtained by the approach explained in full detail above is denatured with a conventional method, and it fixes on a microplate, and considers as Sample DNA (this may be called "solid phase-ized DNA" below). the approach same on the other hand as the above -- DNA of "the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a known epidemia enterovirus separation stock, and the human serum protein type of enterovirus" -- magnification -- and an indicator can be carried out and it can consider as the DNA probe for human serum protein type discernment. The indicator of this DNA probe for human serum protein type discernment changes and uses for Biotin dUTP a part of dTTP used for example, for a DNA magnification reaction, and can carry it out easily by performing DNA magnification.

[0012] the solid phase-ized DNA (sample DNA) above-mentioned after denaturing various kinds of DNA probes for human serum protein type discernment obtained in this way -- in addition, the human serum protein type of enterovirus used for preparation of solid phase-ized DNA (sample DNA) is discriminable by carrying out hybridization under said **** conditions, and detecting the class and amount of the DNA probe for human serum protein type discernment which were combined to solid phase-ized DNA using enzyme-labeling avidin etc.

[0013]

[Example] Hereafter, an example is given and this invention is further explained to a detail.

Example 1 It experimented using the 31 following kinds of human serum protein type picornavirus standard stocks by which subculture is carried out in detection of a picornavirus standard stock, and (Discernment A) use microorganism National Institute of Health of a human serum protein type. Each of such picornaviruses is standard stocks with which the human serum protein type is identified by the protection test which used the specific antiserum.

[0014]

[Table 1]

株名(血清型)		略号
コクサッキーA群ウイルス	2型	A 2
"	3 "	A 3
"	4 "	A 4
"	8 "	A 8
"	9 "	A 9
コクサッキーB群ウイルス	1型	B 1
"	2 "	B 2
"	3 "	B 3
"	4 "	B 4
"	5 "	B 5
"	6 "	B 6
エコーウイルス	3型	E 3
"	4 "	E 4
"	5 "	E 5
"	6 "	E 6
"	9 "	E 9
"	1 1 "	E 1 1
"	1 4 "	E 1 4
"	1 6 "	E 1 6
"	1 8 "	E 1 8
"	1 9 "	E 1 9
"	2 4 "	E 2 4
"	2 5 "	E 2 5
"	2 7 "	E 2 7
"	3 0 "	E 3 0
エンテロウイルス	7 1型	E 7 1
ボリオウイルス	1型	P V 1
"	2 "	P V 2
"	3 "	P V 3
ライノウイルス	3型	R H 3
"	7 "	R H 7

[0015] (B) The precipitate after settling extract above-mentioned each virus liquid of RNA by ultracentrifuge actuation by shoe cloth 15% It collected in Tris-EDTA, the phenol/chloroform extraction was performed, and ethanol precipitate was performed.

(C) cDNA which originates in each virus using a reverse transcriptase (Bthesda Research Laboratories) by using as mold each RNA obtained by the synthetic aforementioned (B) term of cDNA was compounded.

[0016] (D) the primer pair which can amplify the gene of the picornavirus of the synthetic aforementioned (A) term of the primer for PCR in common -- a human serum protein type -- the following array (1) which has a complementarity to each of a 5'-untranslation region and ****2 field based on the base sequence of the gene field which carries out the code of the ****4 and ****2 protein with a specific base sequence, and array (2) CTACTTGGGTGTCCGTGTT (1) TGGTGGTGGAAAGTTGCCTGA (2)

the primer of 20 bases shown by ***** -- phospho friend DAITO (Phosphoramidite) -- law -- Applied Biosystem make and model 381-A It compounded using the DNA synthesis machine, refined using the OPCTM cartridge, and was used as a primer of PCR.

[0017] (E) Magnification of the gene for solid phase-ized DNA preparation (sample DNA) (PCR) As reaction mixture, it is 10X. Buffer-solution (Reaction Buffer) 10microl for a reaction, Deoxy nucleotide 3-phosphoric-acid mixed liquor (dATP, dCTP, dGTP, and dTTP; 1.25 mM each content) 16microl, The above-mentioned synthetic primer (1) (50microM) 2.0microl and the above-mentioned synthetic primer (2) (50microM) 2.0microl, Picornavirus cDNA compounded by the aforementioned (C) term Distilled water is added to 100ng-1microg and Taq polymerase (TAKARA SHUZO make) 1microl

(5Unit), and it is total 100microl. What was carried out was prepared. The denaturation process of a base acid was set up for 95-degree-C 30 seconds, it set up 1 minute and 45-degree-C base chain expanding process for the annealing process in 72-degree-C 2 minutes, and 1 cycle amplified 35 cycles of targets DNA using the amplification system (amplification system; SHITASU). This magnification gene was used as a sample DNA for solid-phase-izing.

[0018] (F) Magnification of the gene for DNA probe preparation for human serum protein type discernment (PCR)

It is 10X as reaction mixture. Buffer-solution (Reaction Buffer) 10microfor reaction 1, Deoxy nucleotide 3-phosphoric-acid mixed liquor (dATP, dCTP, and dGTP; 1.25 mM(s)each dTTP; 0.94 mM) 16microl, Biotin-11-dUTP(Enzo Diagnostics)16.7microl, The above-mentioned synthetic primer (1) (50microM) 2.0microl and the above-mentioned synthetic primer (2) (50microM) 2.0microl, Picornavirus cDNA100ng-1microg compounded by the aforementioned (C) term It reaches, distilled water is added to Taq polymerase (TAKARA SHUZO) 1microl (5Unit), and it is total 100microl. What was carried out was prepared. The denaturation process of a base chain is set up for 95-degree-C 30 seconds, it sets up 1 minute and 45-degree-C base chain expanding process for an annealing process in 72-degree-C 2 minutes, and 1 cycle is amplification. 35 cycles of targets DNA were amplified using the system (SHITASU). The gene DNA by which the indicator was carried out by this biotin was used as a DNA probe for human serum protein type discernment.

[0019] (G) The ethidium bromide was added to agarose gel of 3.0% of checks of the magnification gene DNA by gel electrophoresis ml 0.5microg /, and electrophoresis of DNA amplified by the above (E) and the (F) term was performed. 254nm ultraviolet rays were irradiated after migration, the coloring reaction of the ethidium bromide detected the DNA band, and the target DNA band of about 650 bases originating in the gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in the part and human serum protein type of a 5'-untranslation region of enterovirus was checked.

(H) The gene DNA amplified by purification of Magnification DNA, the density measurement above (E), and the (F) term was settled after an extract and using ethanol under a phenol/chloroform, it collected, and concentration was computed with the absorbance of 260nm.

[0020] (I) Plate hybridization microplate solid phase technique (Inouye

Hondo.J.Cli.Microbiol.28:1469.1990) It carried out by the strange method. They are 1.5M NaCl, 10mM sodium phosphate, and 10mM after thermal denaturation and about 50ng / 100microl/well in the sample DNA refined by the above-mentioned (H) term. It is a microplate (NUNC-IMMUNO PLATE MAXISORP F96) under EDTA existence. It solid-phase-ized in 37-degree-C 2 hours. This was washed 3 times by PBS-Tween 20, and the unreacted sample DNA was removed. Hybridization performed 1.25ng / 50 degree C of 100microl/well for the DNA probe for human serum protein type discernment refined by the aforementioned (H) term to said microplate after thermal denaturation for 8 hours under 50% formamide, 0.75MNaCl, 0.1%Tween 20, and Salmon sperm 50microg/ml existence. The microplate was washed 3 times by PBS-Tween 20 after hybridization, and the DNA probe for unreacted human serum protein type discernment was removed. next, 1:1,000 diluent (1%BSA, 0.1% Triton X-100, and PBS-Tween 20) of peroxidase-labeling streptoavidin -- dropping -- it was made to react for room temperature 2 hours It is after 3 times washing, 0.012%H₂O₂ and 0.04% alt.phenylenediamine, and 0.05/0.024M at PBS-Tween 20 about a microplate again. An sodium phosphate-citric acid (pH5.0) is made to react in the state of protection from light at a room temperature in addition for 30 minutes so that it may become 100microl/well, and it is 4Ns. 50micro l/well of sulfuric acids was added, and the reaction was stopped. The absorbance (OD) was measured for the amount of coloring of the microplate produced by the reaction on the wavelength of 492nm using the microplate reader (Biorad make). It asked for the binding fraction (%) of the DNA probe for human serum protein type discernment from the absorbance of each microplate as follows.

Binding fraction (%) =(OD value of hybridization of solid phase-ized DNA [of the OD value / same human serum protein type virus origin of the hybridization of a solid phase-ized DNA of the human serum protein type virus origin and the DNA probe for discernment which are different in **], and

DNA probe for discernment) x100.

The result is shown in the 1st table. In addition, each null column in the 1st table is the value of less than 10% of association.

[0021]

[Table 2]

第1表
標準の型鑑別(結合率: %)

	A2	A3	A4	A5	A9	B1	B2	B3	B4	B5	B6	E3	E4	E5	E6	E9	E11	E14	E16	E18	E19	E24	E25	E27	E30	E71	PV1	PV2	PV3	RH3	RH7
A2	100																														
A3		100																													
A4			100																												
A8				100																											
A9					100																										
相						100																									
B1							100																								
B2								100																							
B3									100																						
B4										100																					
B5											100																				
B6												100																			
IC													100																		
E3														100																	
E4															100																
E5																100															
E6																	100														
D																		100													
E9																			100												
E11																				100											
E14																					100										
E16																						100									
N																							100								
E18																								100							
E19																									100						
E24																										100					
E25																											100				
A																												100			
E27																															
E30																															
E71																															
PV1																															
PV2																															
PV3																															
RH3																															
RH7																															

[0022] (J) The amplified target DNA band was detected by the gel electrophoresis after PCR about results and all the **** picornavirus standard stocks for consideration (31 shares). Moreover, as a result of performing plate hybridization, the cross reaction was not accepted between the magnification DNA of each human serum protein type origin as the joint pattern shown in the 1st table. It became clear from this joint pattern for detection of enterovirus and discernment of each human serum protein type to be possible.

[0023] Example 2 It experimented using the enterovirus separation stock with which it dissociated from the patient of (Discernment A) use microorganism following of detection of an enterovirus separation stock, and a human serum protein type, and the human serum protein type was identified by the protection test using the specific antiserum, and the standard stock of an example 1.

(1) Enterovirus separation stock [Table 3]

株名 (血清型)	分離時期
コクサッキーA群ウイルス4型 (A 4)	
1 1 5 5 / 7 2	1 9 7 2年
1 3 6 1 / 8 2	1 9 8 2年
0 2 6 9 / 8 4	1 9 8 4年
0 0 2 5 / 8 6	1 9 8 6年
0 0 2 3 / 8 7	1 9 8 7年
0 4 0 6 / 8 9	1 9 8 9年
0 3 1 3 / 9 1	1 9 9 1年
エコーウイルス11型 (E 1 1)	
1 0 3 6 / 7 1	1 9 7 1年
1 1 8 3 / 7 7	1 9 7 7年
1 1 4 9 / 8 7	1 9 8 7年
3 1 3 7 / 8 1	1 9 8 1年
1 3 0 3 / 8 3	1 9 8 3年
0 7 9 8 / 8 4	1 9 8 4年
0 4 0 0 / 8 5	1 9 8 5年
0 1 0 7 / 9 0	1 9 9 0年
エンテロウイルス71型 (E 7 1)	
ナゴヤ / 7 0	1 9 7 0年
3 0 5 9 / 7 8	1 9 7 8年
3 3 5 9 / 8 3	1 9 8 3年
4 1 3 2 / 8 5	1 9 8 5年
2 3 6 a / 8 6	1 9 8 6年
2 3 6 c / 8 6	1 9 8 6年
0 2 5 3 / 8 6	1 9 8 6年
2 5 8 7 / 8 9	1 9 8 9年
4 0 9 4 / 9 0	1 9 9 0年

[0024] (2) Standard stock [Table 4]

コクサッキーA群ウイルス	4型 (A 4)
コクサッキーB群ウイルス	2 (B 2)
〃	3 (B 3)
〃	5 (B 5)
エコーウイルス	9 (E 9)
〃	1 1 (E 1 1)
〃	3 0 (E 3 0)
エンテロウイルス	7 1 (E 7 1)
ポリオウイルス	3 (P V 3)

[0025] (B) the experiment approach and the approach of each virus of the result above to the example 1

given in (B) term -- RNA -- extracting -- an approach given in (** C) term -- every -- cDNA was compounded. Furthermore, as a result of amplifying the gene for solid phase-ized DNA preparation by the approach given in (** E) term, amplifying the gene for DNA probe preparation for human serum protein type discernment by the approach given in (** F) term and performing gel electrophoresis given in (** G) term about these magnification genes DNA, the magnification gene DNA band originating in all the used stocks has been checked. After refining these magnification gene DNA by the approach given in (** H) term and performing density measurement, plate hybridization was carried out like (** I) term publication, and the binding fraction (%) of each probe was computed. The result is shown in the 2nd table - the 4th table. In addition, the binding fraction of the null column of front Naka is 10% or less of value.

[0026]

[Table 5]

第 2 表
コクサッキー A 群ウイルス 4 型 (A 4) 分離株の型鑑別 (結合率 : %)

			血清型識別用 DNA プローブ							
			1155/72	1361/82	0269/84	0025/86	0023/87	0406/89	0313/91	標準株 A4
固 相 分 離 化 株	A 4 0269/84 0025/86 0023/87 0406/89 0313/91	1155/72	100							
		1361/82		100						
		0269/84			100	63	50	50	58	
		0025/86			81	100	60	43	50	
		0023/87			50	44	100	36	33	
		0406/89			56	44	36	100	100	
		0313/91			56	44	29	79	100	
D N A 株	標準株	A4								100
		B2								
		B3								
		B5								
		E9								
		E11								
		E30								
		E71								
		PV3								

[0027]

[Table 6]

第3表
エコーウイルス11型 (E11) 分離株の型鑑別 (結合率: %)

		血清型識別用 DNA プローブ								
		1036/71	1183/77	1149/78	3137/81	1303/83	0798/84	0400/85	0107/90	標準株E11
固 相 化 D	E 11 分 離 株	1036/71 1183/77 1149/78 3137/81 1303/83 0798/84 0400/85 0107/90	100 100 43 33 20 33 23	100 23 20 93 20 67	20 100 73 100 80 78	22 111 100 100 93 78	20 103 76 100 93 62	20 117 108 104 76 79	20 117 108 104 76 79	92 81 81 81 77 100
N	A 標 準 株	A4 B2 B3 B5 E9 E11 E30 E71 PV3							100	

[0028]
[Table 7]

第4表 テンソロジーイリコツ1型(五71)分離性の別鑑別(検合率: %)

[0029] The cross reaction was not accepted between the same human serum protein types between solid phase-ized DNA of the standard stock origin of all the DNA probes and each human serum protein types of the used enterovirus separation stock a passage clear from the joint pattern shown in the 2nd table - the 4th table. On the other hand, about the separation stock in each human serum protein type, the high cross reaction was accepted on the epidemia viral isolation stock (between the same human serum protein types) separated within about ten years. The gene field where a human serum protein type has a

specific base sequence in said human serum protein type of a known epidemia enterovirus separation stock (stock separated within about ten years) from the above result was amplified, and when performing hybridization using the DNA probe for human serum protein type discernment obtained, it became clear for detection of epidemia enterovirus and discernment of a human serum protein type to be easily possible.

[Translation done.]

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TECHNICAL FIELD

[Industrial Application] This invention detects enterovirus to high sensitivity, and relates to the approach of identifying a human serum protein type.

[Translation done.]

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PRIOR ART

[Description of the Prior Art] It is difficult to presume the virus which the enterovirus (Enterovirus) belonging to the Picornaviridae (Picornaviridae) is classified into about 70 kinds of human serum protein types, and the rhinovirus (Rhinovirus) which similarly belongs to the Picornaviridae is classified into about 100 kinds of human serum protein types, shows a variegated infectious disease, and becomes a cause from a clinical manifestation. Therefore, separation identification of a virus is needed for deciding a pathogen. However, a current enterovirus separation method of identification separates a virus using cultivation, and the protection test is further needed for identification. And two - four weeks is required for the isolation culture of these viruses. The separation stock which the protection test which furthermore used the neutralization antiserum of a standard stock cannot human serum protein type judge appears frequently. This is considered for the gene of enterovirus to vary extremely in a nature at high speed, and production of the antiserum which always neutralizes a fresh separation stock is needed for these solutions. In chlamydia (Chlamydia), the approach of detecting for a short time, using a DNA probe as a direct detection method of the pathogen of an infectious disease is established. However, the detection sensitivity is low, and in enterovirus, in order that the amount of viruses required for the probe method may not be obtained from a patient specimen but the gene of enterovirus may vary to a high speed extremely still like previous statement, the difficulty of identification is expected with the oligo probe of a standard stock. High sensitivity and polymerase chain reaction method [Polymerase Chain Reaction which amplifies DNA specifically Law, ; which writes this as the "PCR method" below, after Saiki et al., Science, 230 volumes, p1350-1354, and 1985 reference] are developed the PCR method using a primer complementary to the base sequence of a 5'-untranslation region, and 5' -- by the PCR method using the primer which has a complementarity in the base sequence of the gene field which carries out the code of the ****4 and ****2 protein in - untranslation region [Rotbart. by which enterovirus is detected H. and S.J. -- Clinical microbiology. and 28 438-442 (1990); Olive.D., M., S.J. general Virology., 71, and 2141-2147 (1990) --]. However, these approaches cannot identify the human serum protein type of enterovirus, therefore enterovirus is detected in a higher precision and the approach of judging a human serum protein type is searched for.

[Translation done.]

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MEANS

[Means for Solving the Problem] this invention persons have a specific base sequence in a part of 5'-untranslation region of enterovirus, and the human serum protein type of enterovirus. Detection [high sensitivity picornaviruses /, such as enterovirus,] is possible by amplifying a field including the gene field which carries out the code of some ****4 and ****2 proteins, and detecting this magnification gene DNA, Furthermore, a human serum protein type uses a known epidemia enterovirus separation stock for this magnification gene DNA, and the same field as the above is combined under the produced DNA probe which amplified and carried out the indicator, and **** conditions. By detecting the joint indicator DNA and analyzing the class of united probe, it finds out that discernment of the highly precise human serum protein type of enterovirus is possible, and came to complete this invention.

[0005] According to this invention, in this way A part of 5'-untranslation region of 1. (i) enterovirus The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. The gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in a part of 5'-untranslation region of enterovirus and the human serum protein type of enterovirus is amplified. (ii) The method of detecting the enterovirus characterized by detecting this magnification gene DNA, 2. A part of 5'-untranslation region of an enterovirus separation stock with the strange (i) human serum protein type The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. The gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a strange enterovirus separation stock and the human serum protein type of enterovirus is amplified. This magnification gene DNA is solid-phase-ized to a microplate. A part of 5'-untranslation region of the separation stock of the epidemia enterovirus of known [human serum protein type / (ii)] The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. And carry out an indicator and it considers as the DNA probe for human serum protein type discernment. the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a known epidemia enterovirus separation stock, and the human serum protein type of enterovirus -- magnification -- A DNA probe is added to the DNA solid phase-ized microplate of the above (i). (iii) The human serum protein type discernment approach of the enterovirus which is made to carry out hybridization under **** conditions, and is characterized by analyzing the class of joint probe, 3. The oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of (i) enterovirus and some ****4 and ****2 proteins is the following array (1).

CTACTTGGGTGTCCGTGTT (1)

The oligonucleotide which comes out, has the base sequence shown and has a complementarity in a

down-stream mold intersection is the following array. TGGTGGTGGAAAGTTGCCTGA (2)

Detection or the discernment approach of the above 1 or the enterovirus of 2 characterized by being the oligonucleotide which comes out and has the base sequence shown is offered.

[0006] Detection and the discernment approach of the enterovirus of this invention are further explained to a detail below. a particle symmetrical with the regular icosahedron of the ether resistance in which "picornavirus" does not have an envelope in this specification -- it is -- the diameter of 20-30nm -- it is -- a core -- single stranded RNA -- having -- the molecular weight of this RNA -- about 2.5×10^6 it is -- the virion which has infectivity and has the function of mRNA is meant. moreover, "enterovirus" -- the above-mentioned Picornaviridae -- belonging -- and pH3.0 -- stable -- the buoyant density in the inside of CsCl -- 1.32-1.35g/cm³ it is -- virion is meant and the Coxsackie A group virus, the Coxsackie B group virus, echovirus, enterovirus, a poliovirus, etc. are included by this enterovirus. further -- "rhinovirus" -- the above-mentioned Picornaviridae -- belonging -- and pH3.0 -- unstable -- the buoyant density in the inside of CsCl -- 1.38 - 1.40 g/cm² it is -- virion is meant. One description of this invention is to identify the human serum protein type of this enterovirus while detecting enterovirus by analyzing the class of probe which the human serum protein type increased a part of gene of the strange enterovirus separation stock origin, made combine by the hybridization under the DNA probe and the **** conditions that magnification and the human serum protein type which carried out the indicator produced the same field from the gene of a known epidemia enterovirus separation stock, and combined. Such an approach enables it to identify the human serum protein type of enterovirus, while enterovirus is detectable in a high precision.

[0007] Since a close relationship [a human serum protein type / between those with about 70 sorts, and each human serum protein type], as for enterovirus, it is desirable to use the hybridization under the **** conditions which discernment of a human serum protein type is difficult, and are used by this invention on the occasion of discernment of a human serum protein type on the usual hybridization conditions. Here, the hybridization under **** conditions means the hybridization under existence of a formamide. Especially the abundance of the formamide in this hybridization condition usually has 40 - 60% of desirable within the limits 20 to 70%, and especially reaction temperature has desirable within the limits of 40-60 degrees C 40-70 degrees C. Although there is especially no limit in reaction time, within the limits of 1 - 24 hours is usually suitable. Although a standard stock and a separation stock (they are a vaccine stock and a decomposition stock in the case of a poliovirus) will be distinguished in the same human serum protein type and discernment of the human serum protein type of a separation stock is impossible in the hybridization under the above-mentioned **** conditions As a source of enterovirus gene DNA for the DNA probe creation for human serum protein type discernment The DNA probe for human serum protein type discernment by which the human serum protein type was created using the known epidemia enterovirus separation stock (namely, enterovirus stock which was in fashion and was separated within the past ten years) is used. It becomes discriminable [detection of each enterovirus, and a human serum protein type] by performing hybridization under the above-mentioned **** conditions, and analyzing a joint pattern.

[0008] Magnification of a gene field including the human serum protein type specific base sequence of enterovirus, i.e., "the gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in a part of 5'-untranslation region of enterovirus and the human serum protein type of enterovirus", can be performed as follows. First, the isolation culture stock from clinical specimens, such as cerebrospinal fluid extracted at the time of a medical examination, and a clinical specimen and the human serum protein type by which subculture is carried out extract RNA from a known enterovirus standard stock etc. with a conventional method, and produces cDNA for this extract RNA using reverse transcriptase. The die length which includes the gene field which carries out the code of the 5'-untranslation region of enterovirus, and ****4 and ****2 for the oligonucleotide which has a human serum protein type specific base sequence, i.e., "the oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of an enterovirus separation stock and some ****4 and ****2 proteins, and a down-stream mold intersection", as a primer in this cDNA amplifies the gene DNA field of about

650 bases. the PCR method for which magnification of a gene is usually used -- [-- JP,61-274697,A, JP,62-281,A, 239 Sakai sScience(s), and p487-491 reference] can perform the detail of this PCR method easily.

[0009] On the occasion of magnification of a gene field including the human serum protein type specific base sequence of enterovirus, as an oligonucleotide which can be used as a primer The oligonucleotide which has a complementarity in the mold intersection of the upstream of a gene field including a human serum protein type specific base sequence, and a down-stream mold intersection, Namely, if "the oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of enterovirus and some ****4 and ****2 proteins, and a down-stream mold intersection" is used for coincidence You may be what kind of oligonucleotide. It is appropriate to use as a primer the oligonucleotide which was specific to enterovirus, and set the high base sequence of similarity as the 5'-untranslation region (upstream mold intersection) and ****2 field (down-stream mold intersection) between seeds, and carried out chemosynthesis in them based on the base sequence based on desirable known human serum protein type specific base sequence data.

[0010] As the primer which carried out chemosynthesis, i.e., an oligonucleotide which has a complementarity in the mold intersection of the upstream of an enterovirus specific gene field, it is the following array (1).

CTACTTGGGTGTCCGTGTT (1)

The oligonucleotide which has a complementarity in a down-stream mold intersection is the following array (2).

TGGTGGTGGAAAGTTGCCTGA (2)

It is more desirable to use the primer which comes out and has the base sequence shown. The chemosynthesis of the primer mentioned above is model 381-A, the known the nucleic-acid-biosynthesis machine usually used, for example, Applied Biosystem make, in itself. It can carry out easily with the solid phase synthesis method using a DNA synthesis machine etc. Like the above, polyacrylamide gel electrophoresis, agarose gel electrophoresis, etc. which are usually used can separate, and the gene field DNA including the human serum protein type specific base sequence of the enterovirus which was carried out and was amplified by the PCR method can be detected as a band, and, thereby, can check the gene DNA of the enterovirus origin. In addition, detection of the DNA band after electrophoresis can be dyed by the ethidium bromide, and UV irradiation can perform it easily.

[0011] DNA of "the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a strange enterovirus separation stock and the human serum protein type of enterovirus" obtained by the approach explained in full detail above is denatured with a conventional method, and it fixes on a microplate, and considers as Sample DNA (this may be called "solid phase-ized DNA" below). the approach same on the other hand as the above -- DNA of "the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a known epidemia enterovirus separation stock, and the human serum protein type of enterovirus" -- magnification -- and an indicator can be carried out and it can consider as the DNA probe for human serum protein type discernment. The indicator of this DNA probe for human serum protein type discernment changes and uses for Biotin dUTP a part of dTTP used for example, for a DNA magnification reaction, and can carry it out easily by performing DNA magnification.

[0012] the solid phase-ized DNA (sample DNA) above-mentioned after denaturing various kinds of DNA probes for human serum protein type discernment obtained in this way -- in addition, the human serum protein type of enterovirus used for preparation of solid phase-ized DNA (sample DNA) is discriminable by carrying out hybridization under said **** conditions, and detecting the class and amount of the DNA probe for human serum protein type discernment which were combined to solid phase-ized DNA using enzyme-labeling avidin etc.

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EXAMPLE

[Example] Hereafter, an example is given and this invention is further explained to a detail. Example 1 It experimented using the 31 following kinds of human serum protein type picornavirus standard stocks by which subculture is carried out in detection of a picornavirus standard stock, and (Discernment A) use microorganism National Institute of Health of a human serum protein type. Each of such picornaviruses is standard stocks with which the human serum protein type is identified by the protection test which used the specific antiserum.

[0014]

[Table 1]

株名（血清型）		略号
コクサッキーA群ウイルス	2型	A 2
〃	3〃	A 3
〃	4〃	A 4
〃	8〃	A 8
〃	9〃	A 9
コクサッキーB群ウイルス	1型	B 1
〃	2〃	B 2
〃	3〃	B 3
〃	4〃	B 4
〃	5〃	B 5
〃	6〃	B 6
エコーウイルス	3型	E 3
〃	4〃	E 4
〃	5〃	E 5
〃	6〃	E 6
〃	9〃	E 9
〃	11〃	E 1 1
〃	14〃	E 1 4
〃	16〃	E 1 6
〃	18〃	E 1 8
〃	19〃	E 1 9
〃	24〃	E 2 4
〃	25〃	E 2 5
〃	27〃	E 2 7
〃	30〃	E 3 0
エンテロウイルス	71型	E 7 1
ポリオウイルス	1型	P V 1
〃	2〃	P V 2
〃	3〃	P V 3
ライノウイルス	3型	R H 3
〃	7〃	R H 7

[0015] (B) The precipitate after settling extract above-mentioned each virus liquid of RNA by ultracentrifuge actuation by shoe cloth 15% It collected in Tris-EDTA, the phenol/chloroform extraction was performed, and ethanol precipitate was performed.

(C) cDNA which originates in each virus using a reverse transcriptase (Bthesda Research Laboratories) by using as mold each RNA obtained by the synthetic aforementioned (B) term of cDNA was compounded.

[0016] (D) the primer pair which can amplify the gene of the picornavirus of the synthetic aforementioned (A) term of the primer for PCR in common -- a human serum protein type -- the following array (1) which has a complementarity to each of a 5'-untranslation region and ****2 field based on the base sequence of the gene field which carries out the code of the ****4 and ****2 protein with a specific base sequence, and array (2) CTACTTGGGTGTCCGTGTT (1)

TGGTGGTGGAAAGTTGCCTGA (2)

the primer of 20 bases shown by ***** -- phospho friend DAITO (Phosphoramidite) -- law -- Applied Biosystem make and model 381-A It compounded using the DNA synthesis machine, refined using the OPCTM cartridge, and was used as a primer of PCR.

[0017] (E) Magnification of the gene for solid phase-ized DNA preparation (sample DNA) (PCR) As reaction mixture, it is 10X. Buffer-solution (Reaction Buffer) 10microl for a reaction, Deoxy nucleotide 3-phosphoric-acid mixed liquor (dATP, dCTP, dGTP, and dTTP; 1.25 mM each content) 16microl, The above-mentioned synthetic primer (1) (50microM) 2.0microl and the above-mentioned synthetic primer (2) (50microM) 2.0microl, Picornavirus cDNA compounded by the aforementioned (C) term Distilled water is added to 100ng-1microg and Taq polymerase (TAKARA SHUZO make) 1microl (5Unit), and it is total 100microl. What was carried out was prepared. The denaturation process of a base acid was set up for 95-degree-C 30 seconds, it set up 1 minute and 45-degree-C base chain expanding process for the annealing process in 72-degree-C 2 minutes, and 1 cycle amplified 35 cycles of targets DNA using the amplification system (amplification system; SHITASU). This magnification gene was used as a sample DNA for solid-phase-izing.

[0018] (F) Magnification of the gene for DNA probe preparation for human serum protein type discernment (PCR)

It is 10X as reaction mixture. Buffer-solution (Reaction Buffer) 10microfor reaction 1, Deoxy nucleotide 3-phosphoric-acid mixed liquor (dATP, dCTP, and dGTP; 1.25 mM(s)each dTTP; 0.94 mM) 16microl, Biotin-11-dUTP(Enzo Diagnostics)16.7microl, The above-mentioned synthetic primer (1) (50microM) 2.0microl and the above-mentioned synthetic primer (2) (50microM) 2.0microl, Picornavirus cDNA100ng-1microg compounded by the aforementioned (C) term It reaches, distilled water is added to Taq polymerase (TAKARA SHUZO) 1microl (5Unit), and it is total 100microl. What was carried out was prepared. The denaturation process of a base chain is set up for 95-degree-C 30 seconds, it sets up 1 minute and 45-degree-C base chain expanding process for an annealing process in 72-degree-C 2 minutes, and 1 cycle is amplification. 35 cycles of targets DNA were amplified using the system (SHITASU). The gene DNA by which the indicator was carried out by this biotin was used as a DNA probe for human serum protein type discernment.

[0019] (G) The ethidium bromide was added to agarose gel of 3.0% of checks of the magnification gene DNA by gel electrophoresis ml 0.5microg /, and electrophoresis of DNA amplified by the above (E) and the (F) term was performed. 254nm ultraviolet rays were irradiated after migration, the coloring reaction of the ethidium bromide detected the DNA band, and the target DNA band of about 650 bases originating in the gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in the part and human serum protein type of a 5'-untranslation region of enterovirus was checked.

(H) The gene DNA amplified by purification of Magnification DNA, the density measurement above (E), and the (F) term was settled after an extract and using ethanol under a phenol/chloroform, it collected, and concentration was computed with the absorbance of 260nm.

[0020] (I) Plate hybridization microplate solid phase technique (Inouye Hondo.J.Cli.Microbiol.28:1469.1990) It carried out by the strange method. They are 1.5M NaCl, 10mM

sodium phosphate, and 10mM after thermal denaturation and about 50ng / 100microl/well in the sample DNA refined by the above-mentioned (H) term. It is a microplate (NUNC-IMMUNO PLATE MAXISORP F96) under EDTA existence. It solid-phase-ized in 37-degree-C 2 hours. This was washed 3 times by PBS-Tween 20, and the unreacted sample DNA was removed. Hybridization performed 1.25ng / 50 degree C of 100microl/well for the DNA probe for human serum protein type discernment refined by the aforementioned (H) term to said microplate after thermal denaturation for 8 hours under 50% formamide, 0.75MNaCl, 0.1%Tween 20, and Salmon sperm 50microg/ml existence. The microplate was washed 3 times by PBS-Tween 20 after hybridization, and the DNA probe for unreacted human serum protein type discernment was removed. next, 1:1,000 diluent (1%BSA, 0.1% Triton X-100, and PBS-Tween 20) of peroxidase-labeling streptoavidin -- dropping -- it was made to react for room temperature 2 hours It is after 3 times washing, 0.012%H₂O₂ and 0.04% alt.phenylenediamine, and 0.05/0.024M at PBS-Tween 20 about a microplate again. An sodium phosphate-citric acid (pH5.0) is made to react in the state of protection from light at a room temperature in addition for 30 minutes so that it may become 100microl/well, and it is 4 Ns. 50micro l/well of sulfuric acids was added, and the reaction was stopped. The absorbance (OD) was measured for the amount of coloring of the microplate produced by the reaction on the wavelength of 492nm using the microplate reader (Biorad make). It asked for the binding fraction (%) of the DNA probe for human serum protein type discernment from the absorbance of each microplate as follows.

Binding fraction (%) =(OD value of hybridization of solid phase-ized DNA [of the OD value / same human serum protein type virus origin of the hybridization of a solid phase-ized DNA of the human serum protein type virus origin and the DNA probe for discernment which are different in **], and DNA probe for discernment) x100.

The result is shown in the 1st table. In addition, each null column in the 1st table is the value of less than 10% of association.

[0021]

[Table 2]

第1表
標準株の型鑑別(結合率: %)

		血清型別用アプローチ										-		7																			
		A2	A3	A4	A5	A9	B1	B2	B3	B4	B5	B6	E3	E4	E5	E6	E9	E11	E14	E16	E18	E19	E24	E25	E30	E71	PV1	PY2	PV3	RH3	RH7		
固	A2	100	100																														
固	A3			100	100																												
相	B1						100	100	100	100	100	100																					
相	B2							100	100	100	100	100																					
相	B3								100	100	100	100																					
相	B4									100	100	100																					
相	B5										100	100																					
相	B6											100																					
化	E3												100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100		
化	E4																																
化	E5																																
化	E6																																
D	E9																																
D	E11																																
D	E14																																
D	E16																																
N	E18																																
N	E19																																
N	E24																																
N	E25																																
A	E27																																
A	E30																																
A	E71																																
PV1																																	
PV2																																	
PV3																																	
RH3																																	
RH7																																	

[0022] (J) The amplified target DNA band was detected by the gel electrophoresis after PCR about results and all the **** picornavirus standard stocks for consideration (31 shares). Moreover, as a result of performing plate hybridization, the cross reaction was not accepted between the magnification DNA of each human serum protein type origin as the joint pattern shown in the 1st table. It became clear from

this joint pattern for detection of enterovirus and discernment of each human serum protein type to be possible.

[0023] Example 2 It experimented using the enterovirus separation stock with which it dissociated from the patient of (Discernment A) use microorganism following of detection of an enterovirus separation stock, and a human serum protein type, and the human serum protein type was identified by the protection test using the specific antiserum, and the standard stock of an example 1.

(1) Enterovirus separation stock [Table 3]

株名 (血清型)	分離時期
コクサッキーA群ウイルス4型 (A 4)	
1155/72	1972年
1361/82	1982年
0269/84	1984年
0025/86	1986年
0023/87	1987年
0406/89	1989年
0313/91	1991年
エコーワイルス11型 (E 11)	
1036/71	1971年
1183/77	1977年
1149/87	1987年
3137/81	1981年
1303/83	1983年
0798/84	1984年
0400/85	1985年
0107/90	1990年
エンテロウイルス71型 (E 71)	
ナゴヤ/70	1970年
3059/78	1978年
3359/83	1983年
4132/85	1985年
236a/86	1986年
236c/86	1986年
0253/86	1986年
2587/89	1989年
4094/90	1990年

[0024] (2) Standard stock [Table 4]

コクサッキーA群ウイルス	4型 (A 4)
コクサッキーB群ウイルス	2 (B 2)
〃	3 (B 3)
〃	5 (B 5)
エコーワイルス	9 (E 9)
〃	11 (E 11)
〃	30 (E 30)
エンテロウイルス	71 (E 71)
ポリオウイルス	3 (P V 3)

[0025] (B) the experiment approach and the approach of each virus of the result above to the example 1 given in (B) term -- RNA -- extracting -- an approach given in (** C) term -- every -- cDNA was compounded. Furthermore, as a result of amplifying the gene for solid phase-ized DNA preparation by the approach given in (** E) term, amplifying the gene for DNA probe preparation for human serum protein type discernment by the approach given in (** F) term and performing gel electrophoresis given in (** G) term about these magnification genes DNA, the magnification gene DNA band originating in

all the used stocks has been checked. After refining these magnification gene DNA by the approach given in (** H) term and performing density measurement, plate hybridization was carried out like (** I) term publication, and the binding fraction (%) of each probe was computed. The result is shown in the 2nd table - the 4th table. In addition, the binding fraction of the null column of front Naka is 10% or less of value.

[0026]

[Table 5]

第 2 表
コクサッキーA群ウイルス4型 (A4) 分離株の型鑑別 (結合率: %)

			血清型識別用DNAプローブ							
			1155/72	1361/82	0269/84	0025/86	0023/87	0406/89	0313/91	標準株A4
固	A	1155/72	100							
	4	1361/82		100						
相		0269/84			100	63	50	50	58	
分		0025/86			81	100	60	43	50	
離		0023/87			50	44	100	36	33	
化		0406/89			56	44	36	100	100	
		0313/91			56	44	29	79	100	
D	N	A	標準株	A4						100
				B2						
				B3						
				B5						
				E9						
				E11						
				E30						
				E71						
				PV3						

[0027]

[Table 6]

第3表
エコーウイルス11型 (E11) 分離株の型鑑別 (結合率: %)

		血清型識別用 DNA プローブ						標準株E11		
		1036/71	1183/77	1149/78	3137/81	1303/83	0798/84	0400/85	0107/90	
固 相 化 D	E	1036/71	100		37	37		33	23	
	11	1149/78		100	20	22		20		
	分	3137/81	43		23	100	111	103	117	92
	離	1303/83	33		20	73	100	76	108	81
	株	0798/84	20			93	100	100	104	81
	化	0400/85	33		20	80	93	76	100	77
N	D	0107/90	23		67	78	62	79	100	
	A	A4								
A	B	B2								
	B	B3								
	B	B5								
	E	E9								
	E	E11								
	E	E30								
P	E	E71								
	V	PV3								
										100

[0028]
[Table 7]

第4表
エンテロウイルス71型(E71)分離株の型鑑別(結合率: %)

		血清型識別用DNAプローブ							標準株E71	
		ナジ /70	3059/78	3359/83	4132/85	236a/86	236c/86	0253/86	2587/89	4094/90
固相分離化	E	ナジ /70	100	110	100	84	98	90	89	
	3059/78	75	100	64	105	75	62	63		
	3359/83	82	85	100	100	82	83	85	22	
	4132/85	79	95	73	100	71	69	63	26	38
	236a/86	82	90	91	84	100	97	93		
	236c/86	89	100	100	58	104	100	100		
	0253/86	82	90	95	84	104	93	100	100	119
	2587/89			32	37				78	100
	4094/90			37					100	119
N	A	A4	B2	B3	B5	E9	E11	E30	E71	PV3
A	標準株									

100

[0029] The cross reaction was not accepted between the same human serum protein types between solid phase-sized DNA of the standard stock origin of all the DNA probes and each human serum protein types of the used enterovirus separation stock a passage clear from the joint pattern shown in the 2nd table - the 4th table. On the other hand, about the separation stock in each human serum protein type, the high cross reaction was accepted on the epidemia viral isolation stock (between the same human serum protein types) separated within about ten years. The gene field where a human serum protein type has a

specific base sequence in said human serum protein type of a known epidemia enterovirus separation stock (stock separated within about ten years) from the above result was amplified, and when performing hybridization using the DNA probe for human serum protein type discernment obtained, it became clear for detection of epidemia enterovirus and discernment of a human serum protein type to be easily possible.

[Translation done.]

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(54)【発明の名称】 エンテロウイルスの検出および識別方法

(57)【要約】

【構成】 (i) エンテロウイルスの5' - 非翻訳領域
の一部、V p 4 と V p 2 蛋白の一部をコードする遺伝子
領域の上流の型共通部分および下流の型共通部分に相補
性を有するオリゴヌクレオチドをプライマーとして用
い、エンテロウイルスの5' - 非翻訳領域の一部とエン
テロウイルスの血清型に特異的な塩基配列を持つV p 4
およびV p 2 蛋白の一部をコードする遺伝子領域を増幅
し、(ii) 該増幅遺伝子DNAを検出することを特徴
とするエンテロウイルスの検出法。

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【特許請求の範囲】

【請求項1】 (i) エンテロウイルスの5' - 非翻訳領域の一部、V p 4とV p 2蛋白の一部をコードする遺伝子領域の上流の型共通部分および下流の型共通部分に相補性を有するオリゴヌクレオチドをプライマーとして用い、エンテロウイルスの5' - 非翻訳領域の一部とエンテロウイルスの血清型に特異的な塩基配列を持つV p 4およびV p 2蛋白の一部をコードする遺伝子領域を増幅し、

(ii) 該増幅遺伝子DNAを検出することを特徴とするエンテロウイルスの検出法。

【請求項2】 (i) 血清型が未知のエンテロウイルス分離株の5' - 非翻訳領域の一部、V p 4とV p 2蛋白の一部をコードする遺伝子領域の上流の型共通部分および下流の型共通部分に相補性を有するオリゴヌクレオチドをプライマーとして用い、血清型が未知のエンテロウイルス分離株の5' - 非翻訳領域の一部とエンテロウイルスの血清型に特異的な塩基配列を持つV p 4およびV p 2蛋白の一部をコードする遺伝子領域を増幅し、該増幅

で示される塩基配列を有し、下流の型共通部分に相補性※※を有するオリゴヌクレオチドが次の配列
TGGTGGTGGAAAGTTGCCTGA (2)

で示される配列を有するオリゴヌクレオチドであることを特徴とする請求項1又は2のエンテロウイルスの検出または識別方法。

【発明の詳細な説明】

【0001】

【産業上の利用分野】 本発明は、エンテロウイルスを高感度に検出し、血清型を識別する方法に関する。

【0002】

【従来の技術】 ピコルナウイルス科 (Picornaviridae) に属するエンテロウイルス (Enterovirus) はおよそ 70 種類の血清型、同じくピコルナウイルス科に属するライノウイルス (Rhinovirus) はおよそ 100 種類の血清型に分類されており、多彩な感染症を示し、臨床症状から原因となるウイルスを推定することは困難である。そのため、病原体を確定するにはウイルスの分離同定が必要となる。しかし、現在のエンテロウイルス分離同定法は、培養法を用いてウイルスを分離し、同定のためには

更に中和試験が必要になる。そしてこれらウイルスの分

10

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* 幅遺伝子DNAをマイクロプレートに固定
(ii) 血清型が既知の流行エンテロウイルスの5' - 非翻訳領域の一部、V p 4とV p 2蛋白の一部をコードする遺伝子領域の上流の型共通部分と下流の型共通部分に相補性を有するオリゴヌクレオチドとして用い、血清型が既知の流行エンテロウイルスの5' - 非翻訳領域の一部とエンテロウイルスの血清型に特異的な塩基配列を持つV p 4蛋白の一部をコードする遺伝子領域を増幅

血清型識別用DNAプローブとし、

(iii) 该DNAプローブを上記(i)のマイクロプレートに加えて、峻厳条件下で洗浄させ、結合プローブの種類を解離とするエンテロウイルスの血清型識別

【請求項3】 (i) エンテロウイルスの5' - 非翻訳領域の一部、V p 4とV p 2蛋白の一部をコードする遺伝子領域の上流の型共通部分に相補性を有するオリゴヌクレオチドが次の配列 (1)

CTACTTTGGGTGTCCGTGTT (1)

で示される塩基配列を有し、下流の型共通部分に相補性※※を有するオリゴヌクレオチドが次の配列

TGGTGGTGGAAAGTTGCCTGA (2)

既述のようにエンテロウイルスの遺伝子変異をするため、標準株のオリゴプローブが予想される。高感度、特異的にDNAリメラーゼ・チエイン・リアクション法 (Chain Reaction 法、以下これを「PCR」) が開発されてから、5' - 非翻訳領域内、V p 4とV p 2蛋白をコードする塩基配列に相補性を有するプライマーを用いて、エンテロウイルスが検出されている (5. J. Clinical microbiology., 28: 43; Olive, D., M., 5 J. general Virology, 7(1990))。しかしながら、これらの方々ウイルスの血清型を識別することができ、高い精度でエンテロウイルスを検出し、別が可能な方法が求められている。

【0003】

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幅し、この増幅遺伝子DNAを検出することによりエンテロウイルス等のピコルナウイルスの高感度な検出が可能であること、さらにこの増幅遺伝子DNAを、血清型が既知の流行エンテロウイルス分離株を用いて前記と同一の領域を増幅および標識して作製したDNAプローブと峻厳条件下で結合させ、結合標識DNAを検出し、結合したプローブの種類を解析することにより、エンテロウイルスの高感度な血清型の識別が可能なことを見いだし、本発明を完成するに至った。

【0005】かくして、本発明によれば、

1. (i) エンテロウイルスの5' - 非翻訳領域の一部、Vp4とVp2蛋白の一部をコードする遺伝子領域の上流の型共通部分および下流の型共通部分に相補性を有するオリゴヌクレオチドをプライマーとして用い、エンテロウイルスの5' - 非翻訳領域の一部とエンテロウイルスの血清型に特異的な塩基配列を持つVp4およびVp2蛋白の一部をコードする遺伝子領域を増幅し、(ii) 該増幅遺伝子DNAを検出することを特徴とするエンテロウイルスの検出法。
2. (i) 血清型が未知のエンテロウイルス分離株の5' - 非翻訳領域の一部、Vp4とVp2蛋白の一部をコードする遺伝子領域の上流の型共通部分および下流の型共通部分に相補性を有するオリゴヌクレオチドをプライ*

CTACTTGGGTGTCCGTGTT (1)

で示される塩基配列を有し、下流の型共通部分に相補性* *を有するオリゴヌクレオチドが、次の配列

TGGTGGTGGAAAGTTGCCTGA (2)

で示される塩基配列を有するオリゴヌクレオチドであることを特徴とする、上記1又は2のエンテロウイルスの検出または識別方法が提供される。

【0006】以下本発明のエンテロウイルスの検出および識別方法について更に詳細に説明する。本明細書において、「ピコルナウイルス」とは、エンベロープのないエーテル耐性の正二十面体対称の粒子で、直徑2.0~3.0nmであり、中心に1本鎖RNAを持ち、このRNAの分子量は約2.5×10⁶であり、感染性を有し、かつmRNAの機能を有するウイルス粒子を意味するものである。また「エンテロウイルス」とは、上記ピコルナウイルス科に属し、かつpH3.0で安定であり、CsCl中での浮上密度が1.32~1.35g/cm³であるウイルス粒子を意味し、このエンテロウイルス層にはコクサ

(3)

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*マーとして用い、血清型が未知のエンテロウイルスの5' - 非翻訳領域の一部とエンテロウイルスに特異的な塩基配列を持つVp4およびVp2蛋白の一部をコードする遺伝子領域を増幅し、DNAをマイクロプレートに固相化し、(i)既知の流行エンテロウイルスの分離株の5' - 非翻訳領域の上流の型共通部分および下流の型共通部分に相補性を有するオリゴヌクレオチドをプライマーとして用い、血清型が既知の流行エンテロウイルスの5' - 非翻訳領域の一部とエンテロウイルスに特異的な塩基配列を持つVp4およびVp2蛋白の一部をコードする遺伝子領域を増幅および標識し、DNAプローブとし、(iii)DNAプローブ(i)のDNA固相化マイクロプレート条件下でハイブリダイゼーションさせ、種類を解析することを特徴とするエンテロウイルスの血清型識別方法。

3. (i) エンテロウイルスの5' - 非翻訳領域の一部、Vp4とVp2蛋白の一部をコードする遺伝子領域の上流の型共通部分に相補性を有するオリゴヌクレオチドが、次の配列(1)

30 ゼーションで結合させ、結合したプローブすることにより、エンテロウイルスを検出し、このエンテロウイルスの血清型を識別する。このような方法により、エンテロウイルスを検出することができると共に、エンテロウイルスの血清型を識別することが可能となる。

【0007】エンテロウイルスは、血清型があり、また各血清型間が近縁なため、ハイブリダイゼーション条件では血清型の識別が困難な場合に際しては、本発明で用いるハイブリダイゼーションを用いるのが好んで、峻厳条件下でのハイブリダイゼーションアミドの存在下でのハイブリダイゼーションである。このハイブリダイゼーション

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ルス分離株（すなわち過去10年以内に流行し分離されたエンテロウイルス株）を用いて作成された血清型識別用DNAプローブを用いて、上記峻厳条件下でハイブリダイゼーションを行い、結合パターンを解析することにより、各エンテロウイルスの検出および血清型の識別が可能となる。

【0008】エンテロウイルスの血清型特異的塩基配列を含む遺伝子領域、すなわち「エンテロウイルスの5'ー非翻訳領域の一部とエンテロウイルスの血清型に特異的な塩基配列を持つV_p4およびV_p2蛋白の一部をコードする遺伝子領域」の増幅は次のとおり行なうことができる。先ず、診察時に採取した臓液等の臨床検体、臨床検体からの分離培養株、継代培養されている血清型が既知のエンテロウイルス標準株等から常法によりRNAを抽出し、この抽出RNAを逆転写酵素を用いcDNAを作製する。このcDNAに血清型特異的塩基配列を有するオリゴヌクレオチド、すなわち「エンテロウイルス分離株の5'ー非翻訳領域の一部、V_p4とV_p2蛋白の一部をコードする遺伝子領域の上流の型共通部分および下流の型共通部分に相補性を有するオリゴヌクレオチド」をプライマーとして用いるのが適当である。エンテロウイルスの5'ー非翻訳領域、V_p4とV_p2をコードする遺伝子領域を含む長さが約650塩基の遺伝子DNA領域を増幅する。遺伝子の増幅は、通常用いられるPCR法（この*

CTACTTTGGGTGTCCGTGTT (1)

下流の型共通部分に相補性を有するオリゴヌクレオチド※ ※が下記配列(2)

TGGTGGTGGAAGTTGCCTGA (2)

で示される塩基配列を有するプライマーを用いるのがより好ましい。上述したプライマーの化学合成は、それ自体既知の通常用いられる核酸合成機、例えばアプライド・バイオシステム社製、モデル381-A DNA合成機等を用いる固相合成法により容易に行なうことができる。上記の如くしてPCR法により増幅したエンテロウイルスの血清型特異的塩基配列を含む遺伝子領域DNAは、通常用いられるポリアクリルアミドゲル電気泳動、アガロースゲル電気泳動等により分離し、バンドとして検出することができ、これによりエンテロウイルス由来の遺伝子DNAを確認することができる。なお電気泳動後のDNAバンドの検出は、エチジウム・プロマイドで染色し、紫外線照射により容易に行なうことができる。

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* PCR法の詳細については、特開昭61号公報、特開昭62-281号公報、Sal 239巻、p487-491参照】により容易に行なう。

【0009】エンテロウイルスの血清型を含む遺伝子領域の増幅に際して、並用することができるオリゴヌクレオチドと特異的塩基配列を含む遺伝子領域の上流および下流の型共通部分に相補性を有する：

10 チド、すなわち「エンテロウイルスの5'ー非翻訳領域の一部、V_p4とV_p2蛋白の一部をコードする遺伝子領域の上流の型共通部分および下流の型共通性を有するオリゴヌクレオチド」を同時に用れば、いかなるオリゴヌクレオチドであれらの中で、好ましくは既知の血清型特異性をもとに、エンテロウイルスに特異的塩基配列をもつて化学合成したオリゴヌクレオチドをプライマーとして用いるのが適当である。

10 【0010】化学合成したプライマー、エンテロウイルス特異的遺伝子領域の上流の型共通部分に相補性を有するオリゴヌクレオチドとしては、

(1)

20 20 CTACTTTGGGTGTCCGTGTT (1)

の一部をコードする遺伝子領域のDNAを標識して血清型識別用DNAプローブとなる。この血清型識別用DNAプローブのは、DNA増幅反応に用いるdTTPのdUTPに変更して用いて、DNA増幅が容易に実施できる。

【0012】かくして得られる各種の血清型識別用DNAプローブを変性させた後、上記固相化DNA（）に加えて、前記峻厳条件下でハイブリダイゼーションさせ、固相化DNAへ結合したDNAプローブの種類および量を、酵素標識用いて検出することにより、固相化DNAの類型に用いたエンテロウイルス

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血清を用いた中和試験で血清型が同定されている標準株である。

【0014】

【表1】

株名(血清型)	略号
コクサッキーA群ウイルス	2型 A 2
〃	3〃 A 3
〃	4〃 A 4
〃	8〃 A 8
〃	9〃 A 9
コクサッキーB群ウイルス	1型 B 1
〃	2〃 B 2
〃	3〃 B 3
〃	4〃 B 4
〃	5〃 B 5
〃	6〃 B 6
エコーウイルス	3型 E 3
〃	4〃 E 4
〃	5〃 E 5
〃	6〃 E 6
〃	9〃 E 9
〃	11〃 E 11
〃	14〃 E 14
〃	16〃 E 16
〃	18〃 E 18
〃	19〃 E 19
〃	24〃 E 24
〃	25〃 E 25
〃	27〃 E 27
〃	30〃 E 30
エンテロウイルス	71型 E 71
ポリオウイルス	1型 P V 1
〃	2〃 P V 2
〃	3〃 P V 3
ライノウイルス	3型 R H 3
〃	7〃 R H 7

* 【0015】 (B) RNAの抽出

上記各ウイルス液を15%シュークロー操作により沈殿させた後、その沈殿物を回収し、フェノール／クロロホルム抽出－アル沈殿を行った。

(C) c DNAの合成

前記(B)項で得た各RNAを錆型としてスクリプターゼ(Bethesda Research L.用いて、各ウイルスに由来するc DNA・

19 【0016】 (D) PCR用プライマー

前記(A)項のビコルナウイルスの遺伝できるプライマーペアを、血清型特異的V p 4及びV p 2蛋白をコードする基配列をもとに、5'－非翻訳領域とVに相補性を有する下記配列(1)および

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*

CTACTTTGGGTGTCCGTGTT (1)

TGGTGGTGGAAGTTGCCTGA (2)

の塩基配列で示される20塩基のプライマーを、ホスホアミダイト(Phosphoramidite)法によりアブライド・バイオシステム社製、モデル381-A DNA合成機を用いて合成し、OPC₁₀カートリッジを用いて精製し、PCRのプライマーとして使用した。

【0017】 (E) 固相化DNA調製用検体子(サンプ

40 C30秒、アニーリング工程を45°C1工程を72°C2分に設定し、アンプリフィケーションシステム(amplification system)にて、標的DNAを35サイクル増幅した。子を固相化用サンプルDNAとして用い、

【0018】 (F) 血清型識別用DNA

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調製した。1サイクルは、塩基鎖の変性工程を95°C 30秒、アニーリング工程を45°C 1分、塩基鎖伸長工程を72°C 2分に設定し、アンプリフィケーションシステム（シータス社）を用いて標的DNAを35サイクル増幅した。このピオチンで標識された遺伝子DNAを血清型識別用DNAプローブとして用いた。

【0019】(G) ゲル電気泳動法による増幅遺伝子DNAの確認

3. 0%のアガロースゲルにエチジウムプロマイドを0.5 μg/ml加え、上記(E)および(F)項で増幅したDNAの電気泳動を行った。泳動後254 nmの紫外線を照射し、エチジウムプロマイドの発色反応によりDNAバンドを検出し、エンテロウイルスの5' - 非翻訳領域の一部と血清型に特異的な塩基配列を持つVP4およびVP2蛋白の一部をコードする遺伝子領域に由来する約650塩基の標的DNAバンドを確認した。

(H) 増幅DNAの精製および濃度測定

前記(E)および(F)項で増幅した遺伝子DNAをフェノール／クロロホルムにて抽出後、エタノールを用いて沈殿させ回収し、濃度を260 nmの吸光度により算出した。

【0020】(I) プレートハイブリダイゼーションマイクロプレート固相法 (Inouye Hondo, J. Clin. Microbiol. 28 : 1459, 1990) の変法により行った。上記(H)項で精製したサンプルDNAを熱変性後、50 ng/100 μl/wellを、1.5M NaCl、10 mMリシン酸ナトリウム、10 mM EDTA存在下でマイクロプレート (NUNC-IMMUNO PLATE MAXISORP F96) に37°C 2時間で固相化した。これをPBS-Tween 20で3回洗浄し未反応サンプルDNAを除去した。ハイブリダイゼーションは前記(H)項で精製した血清型識別用DNAプローブを熱変性後、1.25 ng/100 μl、0.75 M NaCl、0.2 M Salmon sperm 50 μg/mlの存在下でマイクロプレートに50°C 8時間行った。ハイブリダイゼーション後、マイクロプレートをPBS-Tween 20で3回洗浄し、未反応血清型識別用DNAプローブを除去した。次にペルオキシダーゼ標識ストレプトブリオニン (1:1,000希釈液 (1% BSA, 0.1% X-100, PBS-Tween 20) を滴下し、30分間反応させた。再びマイクロプレートをPBS-Tween 20で3回洗浄後、0.012% H₂O₂、0.05% 2-アミノ-2-メチル-1-プロパンスルホン酸ナトリウム-クエン酸 (pH5.0) をとなるように加え、室温で30分、遮光して4N硫酸50 μl/wellを加え反応を終了させて生じたマイクロプレートの着色度をマイクロプレートリーダー (バイオラド社製) で920 nmで吸光度 (OD) を測定した。各ODの吸光度から血清型識別用DNAプローブの結合率 (%) を次のとおり求めた。

結合率 (%) = (互に異なる血清型ウイルスDNAと識別用DNAプローブとのハイブリダイゼーションのOD値 + 同一血清型ウイルス由来と識別用DNAプローブとのハイブリダイゼーションのOD値) × 100。

その結果を第1表に示す。なお、第1表における結合率10%未満の値である。

【0021】

【表2】

標準的の型式別 (標準本 : 86)

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株名(血清型)	分離時期
コクサッキーA群ウイルス4型 (A 4)	
1155/72	1972年
1361/82	1982年
0269/84	1984年
0025/86	1986年
0023/87	1987年
0406/89	1989年
0313/91	1991年
エコーウイルス11型 (E 11)	
1036/71	1971年
1183/77	1977年
1149/87	1987年
3137/81	1981年
1303/83	1983年
0798/84	1984年
0400/85	1985年
0107/90	1990年
エンテロウイルス71型 (E 71)	
ナツヤ/70	1970年
3059/78	1978年
3359/83	1983年
4132/85	1985年
236a/86	1986年
236c/86	1986年
0253/86	1986年
2587/89	1989年
4094/90	1990年

【0024】(2) 標準株

【表4】

コクサッキーA群ウイルス	4型 (A 4)
コクサッキーB群ウイルス	2 (B 2)
ノ	3 (B 3)
ノ	5 (B 5)
エコーウイルス	9 (E 9)
ノ	11 (E 11)
ノ	30 (E 30)
エンテロウイルス	71 (E 71)
ポリオウイルス	3 (PV 3)

【0025】(B) 実験方法および結果

上記の各ウイルスから実施例1の(B)項記載の方法に

よりRNAを抽出し、同(C)項記載のAを合成した。更に同(E)項記載の方A調製用遺伝子を増幅し、同(F)項記型識別用DNAプローブ調製用遺伝子をの増幅遺伝子DNAについて同(G)項検討を行った結果、用いた全ての株に由子DNAバンドが確認できた。これら増を同(H)項記載の方法で精製し、濃度に、同(I)項記載と同様にブレートハーションさせ、各プローブの結合率(%)の結果を第2表～第4表に示す。なお、結合率が10%以下の値である。

【0026】

【表5】

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第2表

コクサッキーA群ウイルス4型 (A4) 分離株の型鑑別 (結合率: %)

			血清型識別用DNAプローブ						
			1155/72	1361/82	0269/84	0025/86	0023/87	0406/89	0313/91
固 相 分 離 化 株	A 4	1155/72	100						
		1361/82		100					
		0269/84			100	63	50	50	58
		0025/86			81	100	60	43	50
		0023/87			50	44	100	36	33
		0406/89			56	44	36	100	100
		0313/91			56	44	29	79	100
D N A 株	標 準 株	A4							
		B2							
		B3							
		B5							
		E9							
		E11							
		E30							
		E71							
		PV3							

【0027】

【表6】

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第3表
エコーウイルス11型(E11)分離株の型鑑別(結合率: %)

		血清型鑑別用DNAプローブ								
		1036/71	1183/77	1149/78	3137/81	1303/83	0798/84	0460/85	0107/90	標準株E11
固 相 化 D	1036/71	100	100	37	37	33	23			
	1183/77		100	20	22	20				
	1149/78		23	100	111	117	92			
	3137/81	43	20	73	100	76	108	81		
	1303/83	83		93	100	100	104	81		
	0798/84	20	20	80	93	76	100	77		
N	0460/85	33		67	78	62	79	100		
	0107/90	23							100	
A		A4	B2	B3	B5	E9	E11	E30	E71	
A									PV3	

【0028】

【表7】

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第4表
エンテロウイルス71型(E71)分離株の型端別(総合率: %)

		血清型識別用DNAプローブ											
		71P/70	3059/78	3359/83	4132/85	236a/86	236c/86	0253/86	2587/89	4094/90	標準株E71		
分離株	型	100	110	100	84	98	90	69	69	63	63		
		3059/78	75	100	64	105	75	62	62	63	63		
71	3359/83	82	85	100	100	82	82	83	85	22	22		
	4132/85	79	95	72	100	71	69	63	63	26	26		
	236a/86	82	90	91	84	100	97	93	93				
	236c/86	89	100	100	58	104	100	100	100				
	0253/86	82	90	95	84	104	93	100	100				
D	2587/89			32	37					100	119		
N	4094/90				37					78	100		
		A4											
A	標準株	B2	B3	B5	E9	E11	E30	E71	Py3				

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